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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OPP OFFICIAL RECORD
HEALTH EFFECTS DIVISION
SCIENTIFIC DATA REVIEWS
EPA SERIES 361

MAY 31 1996

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT **2,4-DICHLOROPHENOXYACETIC ACID - Triisopropanolamine salt (TIPA); Isopropylamine salt (IPA); and Butoxyethyl ester (BEE):** Review of Mutagenicity Studies As Requested by the Agency in a Letter Dated 10/30/93.

FROM: Jess Rowland, M.S, Toxicologist Jess Rowland 5/23/96
Section I, Toxicology Branch II, Health Effects Division (7509C)

TO: Walter Waldrop / Judy Coombs
Product Manager 71
Reregistration Division

THRU: Yiannakis Ioannou, Ph.D., Head Y M. Ioannou 5/23/96
Section I, Toxicology Branch II, Health Effects Division (7509C)

and

Stephanie Irene, Ph.D., Acting Chief Stephanie K Irene 5/30/96
Toxicology Branch II, Health Effects Division (7509C)

DATA PACKAGE IDENTIFICATIONS: Submission: S472693 DP Barcode: D207071

Chemical	PC Code	Caswell No	MRID No.
IPA	**030025**	**315 U**	**43327303 & 43327304**
TIPA	**030035**	**315 AE**	**43327301 & 43327302**
BEE	**030053**	**315 AI**	**43327305**

ACTION REQUESTED: Review of three *in vitro* mammalian chromosomal aberration in rat lymphocyte assays for IPA, TIPA, and BEE and two CHO/HGPRT forward mutation assays for IPA and TIPA salts to satisfy the 1991 Guideline requirement §84-2(2).

RESPONSE: In the attached letter dated September 30, 1993, the Agency accepted the Registrant's (Dow Elanco) commitment to perform CHO/HGPRT forward gene mutation assays and *in vitro* chromosomal aberration assays in rat lymphocytes for IPA, TIPA and BEE of 2,4-D to satisfy the 1991 mutagenicity guidelines §84-(2). In this submission, the Registrant has submitted these studies. A Data Evaluation Record for each of the five studies cited above are attached. The Executive Summaries are provided below.

I. *"Evaluation of 2,4-D Triisopropanolamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes".* Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-008866-017, 1/13/94. **MRID No. 43327301.**

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327301), rat lymphocyte cultures were exposed to a **2,4-D TIPA** (70.9% a.i.), in deionized distilled water for 4 hours at concentrations of 78, 156, 313, 625, 1,250, 2,500, and 5,000 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D TIPA was tested to the limit concentration, 5,000 µg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 39-57% of negative control without S9 activation or 59-86% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. **There were no statistically significant increases in the proportion of aberrant cells over negative control values.**

This study is classified as **acceptable** and satisfies the requirements for the 1991 Guideline § 84-2(2) for an in vitro cytogenetic mutagenicity assay.

II. *"Evaluation of 2,4-D triisopropanolamine salt in the Chinese Hamster Ovary Cell /Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay".* Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID K-008866-018. 1/31/94. **MRID 43327302.**

EXECUTIVE SUMMARY: In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327302) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a **2,4-D TIPA** salt (70.9%) at concentrations of 800, 1,000, 1,250, 2,500, and 5,000 µg/mL with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at the limit dose of 5,000 µg/mL without S9 activation. **There was no evidence of mutagenic effect at any dose level with or without activation.** Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as **acceptable** and satisfies the requirements for the 1991 Guideline § 84-2(2) for *in vitro* mutagenicity (mammalian forward gene mutation) assay.

III. *"Evaluation of 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes".* Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID M-004725-016, 5/27/94. **MRID No. 43327303.**

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327303), rat lymphocyte cultures were exposed to a **2,4-D IPA** (50.2% a.i.), in deionized distilled water for 4 hours at concentrations of 96, 192, 384, 767, 1,534, 3,068, and 6,137 μg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D IPA was tested to 6,137 μg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 28-43% of negative control without S9 activation or 48-53% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. The statistically significant increase in the proportion of aberrant cells at the highest concentration over the negative control value was determined not to be biologically significant since the background aberration frequency for rat lymphocytes can range from 0- 5.5% and the statistical significance was seen only because the concurrent solvent control value was 0%. **Therefore, it was concluded that 2,4-D IPA at upto 3000 μg/mL was not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.**

This study is classified as **acceptable** and satisfies the requirements for the 1991 Guideline § 84-2(2) for *in vitro* cytogenetic mutagenicity data.

IV. *"Evaluation of 2,4- Dichlorophenoxyacetic acid isopropylamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward mutation Assay".* Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID M-004725-017. 5/27/94. **MRID 43327304.**

EXECUTIVE SUMMARY: In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327304) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a **2,4-D IPA salt** (50.2%) at concentrations of 500, 1,000, 1,500, 2,000, and 3,000 μg/mL with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at a dose of 2,500 μg/mL with and without S9 activation. **There was no evidence of a mutagenic effect at any dose level with or without activation.** Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as **acceptable** and satisfies the requirements for FIFRA Test Guideline 84-2 for *in vitro* mutagenicity (mammalian forward gene mutation) data.

V. *"Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes".* Health and Environmental Sciences, Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-007722-022, Study dates: 5/27/94. **MRID No. 43327305.**

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327305), rat lymphocyte cultures were exposed to **2,4-D BEE** (94.6% a.i.), in dimethyl sulfoxide for 4 hours at concentrations of 87.5, 175, 350, 700, and 1,400 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D BEE was tested to the limit of solubility, 1.4 mg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 31-80% of negative control without S9 activation or 61-67% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response.

There were no statistically significant increases in the proportion of aberrant cells over negative control values. However, cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis. In addition, the highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity, while this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay. Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of metabolic activation, based on the results of this study, cannot be made. **It is concluded that a new study should be conducted and the chemical be evaluated at up to a reproducible cytotoxic level.**

This study is classified as **unacceptable** and does not satisfy the requirements for the 1991 Guideline, 84-2 for *in vitro* cytogenetic mutagenicity data.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

9-30-93

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

CERTIFIED MAIL P322-239
Larry E. Hammond
Product Registration Manager
DowElanco
9002 Purdue Rd.
Indianapolis, IN 46268-1189

Dear Mr. Hammond:

Subject: 2,4-D TIPA, IPA and BEE Mouse Micronucleus Assays

The Agency has reviewed supplemental information submitted by DowElanco to upgrade the 2,4-D IPA and BEE Mouse Bone Marrow Micronucleus Test to acceptable, provided a new study is completed for mammalian cells in culture; forward gene mutation assay. A copy of the data review is enclosed for your information.

With your commitment to do a CHO/HGPRT forward gene mutation assay and an in vitro chromosomal aberration assay in rat lymphocytes to satisfy the new 84-2(2) requirement for all three compounds, the Agency has upgraded the classifications for the Ames assays (guideline 84-2a) for 2,4-D TIPA (41388202, 41797901), IPA (41388203, 41797902), and BEE (41388204, 41797903) to acceptable. The Agency has also concluded that sufficient information has been provided to reclassify the 2,4-D IPA and 2,4-D BEE studies to acceptable for satisfying the 84-2(3) data requirement. (Review of 5/4/93 accepted the 2,4-D TIPA study.)

In your letter dated March 25, 1993, you committed to do a CHO/HGPRT forward gene mutation assay and an in vitro chromosomal aberration assay in rat lymphocytes for all three compounds. These studies must be submitted to the Agency within 12 months of the date of receipt of this letter. Failure to provide these data within the time provided may result in the issuance of a Notice of Intent to Suspend affecting your registrations containing 2,4-D TIPA, IPA and BEE.

If you have any further questions concerning this review, please refer them to Judith Coombs, the Case Review Manager for 2,4-D at (703) 308-8046.

Sincerely,

Walter Waldrop for

Lois Rossi, Chief
Reregistration Branch
Special Review and
Reregistration Division (H7508W)

Enclosure

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DATA EVALUATION RECORD

2,4-D; triisopropylamine salt (2,4-D TIPA)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16A (MRID 43327301)

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group
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Dynamac Corporation
2275 Research Boulevard
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Primary Reviewer:
Steven Brecher, Ph.D.

Signature: Steven Brecher
Date: 4/15/96

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William Spangler, Ph.D.

Signature: William J. Spangler
Date: 4/15/96

Project Manager:
William Spangler, Ph.D.

Signature: William J. Spangler
Date: 4/15/96

Quality Assurance:
Reto Engler, Ph.D.

Signature: Reto Engler
Date: 4/15/96

Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist Jess Rowland Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head Y Ioannou Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in rat lymphocytes

Guideline: §84-2(2)

DP BARCODE: D207071 SUBMISSION: S472693

P.C. CODE: 030035 TOX. CHEM. NO.: 315AE

TEST MATERIAL: 2,4-dichlorophenoxyacetic acid **triisopropanolamine salt**

SYNONYMS: 2,4-D **TIPA**

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-D Triisopropanolamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-008866-017, Study dates: 6/3/93-1/13/94. **MRID No. 43327301.** Unpublished.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327301), rat lymphocyte cultures were exposed to a 2,4-D TIPA formulation (70.9% a.i.), in deionized distilled water for 4 hours at concentrations of 78, 156, 313, 625, 1,250, 2,500, and 5,000 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D TIPA was tested to the limit concentration, 5,000 µg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 39-57% of negative control without S9 activation or 59-86% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. **There were no statistically significant increases in the proportion of aberrant cells over negative control values.**

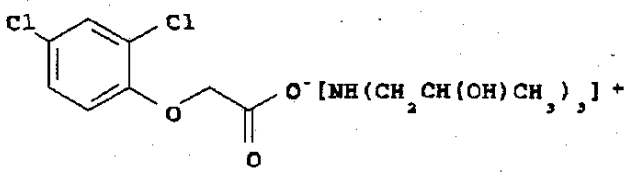
This study is classified as **acceptable** and satisfies the requirements for the 1991 Guideline 84-2(2) for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: 2,4-D TIPA
Description: amber liquid
Lot/Batch #: AGR 295711
Purity: The test material is a formulation intermediate containing 70.9% 2,4-D TIPA salt. The acid (2,4-D) equivalent = 38.0%, TIPA equivalent = 38.6%
Stability of compound: Not addressed
CAS #: 18584-79-7
Structure:



Solvent used: Deionized distilled water
Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:
Negative: Solvent control
Solvent/final concentration: Deionized distilled water/1%
Positive: Nonactivation: Mitomycin C (0.5 µg/mL)
Activation: Cyclophosphamide (6 µg/mL)

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
___ phenobarbital	___ non-induced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other			___ other

S9 purchased from Sitek Research Laboratories, Rockville, Maryland
S9 mix composition: S9 fraction (10% v/v), $MgCl_2$ (10mM), Na_2HPO_4 buffer (50mM), pH 8.0, glucose-6-phosphate (5mM), NADP (4mM), $CaCl_2$ (10mM), KCl (30mM)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

Nonactivated conditions:
Assay 1: 4 hour treatment (78, 156, 313, 625, 1,250, 2,500, 5,000 µg/mL), harvest at 24 hours

Assay 2: 4 hour treatment (1,250, 2,500, 5,000 µg/mL), harvest at 24 and 48 hours

Activated conditions:
Assay 1: 4 hour treatment (78, 156, 313, 625, 1,250, 2,500, 5,000 µg/mL), harvest at 24 hours

Assay 2: 4 hour treatment (1,250, 2,500, 5,000 µg/mL), harvest at 24 and 48 hours

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20 µg/mL).

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? N/A

Cell line or strain periodically checked for karyotype stability? N/A

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5,000 µg/mL, whichever is lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected for subsequent metaphase analysis at 48-hour harvest).

2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to test chemical or positive control for a selected time interval, then the chemical was removed and cells were continued in culture until time of harvest

a. Cell treatment:
Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)

Assay 1: Set 1: 4 hr exposure, -S9, harvest at 24 hrs
Set 2: 4 hr exposure, +S9, harvest at 24 hrs

Assay 2: Set 1: 4 hr exposure, -S9, harvest at 24 hrs
Set 2: 4 hr exposure, +S9, harvest at 24 hrs
Set 3: 4 hr exposure, -S9, harvest at 48 hrs
Set 4: 4 hr exposure, +S9, harvest at 48 hrs

b. Spindle inhibition
Inhibitor used/concentration: Colcemid (0.2 µg/mL)
Administration time: 3 hours (before cell harvest)

c. Cell harvest:
Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).

d. Details of slide preparation:
Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.

e. Metaphase analysis
No. of cells examined per dose: 200
Solvent control: 200
Positive control: 100

Scored for structural: **Yes**

Scored for numerical: **No**

Coded prior to analysis: **Yes**

f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.

g. Statistical analysis: Data evaluated for statistical significance at α=0.01, using Chi-square statistics from two way contingency table.

II. REPORTED RESULTS

Analysis of dose formulations by HPLC indicated that concentrations were between 93 and 103% of target concentrations for Assay 1 and between 100 and 106% for Assay 2.

A. Preliminary cytotoxicity assay: Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, Set 1, without S9 activation, the 5,000 µg/mL concentration reduced the mitotic index to 57% of the negative control value. In Set 2, with S9 activation, 5,000 µg/mL reduced the mitotic index to 86% of the negative control value (first replicate was 54% of control, second replicate was 119% of control).

In Assay 2, Set 1, without S9 activation, 5,000 µg/mL reduced the mitotic index to 39% of the negative control value. In Set 2, with S9 activation, 5,000 µg/mL reduced the mitotic index to 59% of the negative control value. By the 48-hour harvest (Sets 3 and 4), the mitotic indices were similar to negative control values.

B. Cytogenetic assay: Results are presented in Attachments 1 and 2 (study report pages 23, 24, 27, 29).

In Assay 1, cultures treated with 1,250, 2,500, or 5,000 µg/mL of the test compound were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1). 2,4-D TIPA caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory Assay 2, cultures treated with 1,250, 2,500, or 5,000 µg/mL of the test compound were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 2). Cultures treated with 5,000 µg/mL of the test compound were analyzed for chromosomal aberration frequencies at the 48-hour harvest. 2,4-D TIPA caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds.

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. The reviewer agrees with the study authors that 2,4-D TIPA, at up to 5,000 µg/mL, was not clastogenic in cultured rat lymphocytes harvested 24 or 48 hours after treatment (1-2 cell cycles). The negative controls had comparable low frequencies of chromosome aberrations. In addition, the sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls (mitomycin C, -S9, and cyclophosphamide, +S9). The reviewer concludes that the results of this study provided sufficient evidence to consider 2,4-D-TIPA negative in this *in vitro* test system.

B. Study deficiencies

The following deficiences would not be expected to alter the conclusions of the study:

1. Cytotoxicity of 2,4-D TIPA at the limit concentration (5,000 µg/mL) was marginal; 43-61% reduction in mitotic activity versus negative control at the 24-hour harvest without S9 activation, and 14-41% reduction in mitotic activity with S9 activation (14% reduction was mean of duplicate cultures; one exhibited a 46% reduction, the other, a 19% increase).

2. The report indicated that a summary of characterization data for 2,4-D TIPA was included as Table 8. However, Table 8 was not provided.

ATTACHMENTS

TABLE 6C

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D TIPA Positive Control: (6 µg/ml CP)

	Neg. Control[a]			1250 µg/ml			2500 µg/ml			5000µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	5	0	5	3	2	5	2	5	7	2	1	3	3	3	6
Cromosome Gaps	0	1	1	2	0	2	0	0	0	0	0	0	0	2	2
Chromatid Breaks	0	1	1	2	3	5	2	0	2	2	2	4	16	9	25
Chromatid Exchanges	0	0	0	0	0	0	0	2	2	0	0	0	3	5	8
Chromosome Breaks	1	1	2	0	0	0	1	1	2	0	1	1	6	5	11
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4
Total Aberrations	1	2	3	2	3	5	3	3	6	2	3	5	27	23	50
(excluding gaps)[b]			(1.5)			(2.5)			(3.0)			(2.5)			(50.0)
No. of cells with Aberr.	1	2	3	2	3	5	3	3	6	2	3	5	17	16	33[c]
(excluding gaps)[b]			(1.5)			(2.5)			(3.0)			(2.5)			(33.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	3	2	5

[a]1% Deionized Distilled Water.
[b]Values in parentheses are percentages.
[c]Significantly (alpha<0.01) different from the negative control.

Attachment 2, Page 2 of 2

TABLE 4A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D TIPA Positive Control: (0.5 µg/ml MMC)

	Neg. Control[a]			1250 µg/ml			2500 µg/ml			5000 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	0	4	4	3	0	3	5	1	6	7	3	10	10	9	19
Chromosome Gaps	1	1	2	0	0	0	0	1	1	1	0	1	0	0	0
Chromatid Breaks	1	2	3	0	0	0	2	2	4	4	2	6	8	10	18
Chromatid Exchanges	0	0	0	0	0	0	5	0	5	0	1	1	17	17	34
Chromosome Breaks	2	0	2	1	0	1	1	0	1	3	0	3	5	5	10
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total Aberrations	3	2	5	1	0	1	8	2	10	7	3	10	30	37	67
(excluding gaps)[b]			(2.5)			(0.5)			(5.0)			(5.0)			(67.0)
No. of cells with Aberr.	3	2	5	1	0	1	5	2	7	6	5	11	19	22	41[c]
(excluding gaps)[b]			(2.5)			(0.5)			(3.5)			(5.5)			(41.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	2	2	1	1	2

[a]1% Deionized Distilled Water.
[b]Values in parentheses are percentages.
[c]Significantly (alpha<0.01) different from the negative control.

Attachment 1, Page 1 of 2

TABLE 4B

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D TIPA Positive Control: (6 µg/ml CP)

	Neg. Control[a]			1250 µg/ml			2500 µg/ml			5000 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	0	3	3	0	4	4	0	1	1	2	4	6	10	3	13
Chromosome Gaps	2	1	3	0	1	1	2	0	2	0	1	1	1	2	3
Chromatid Breaks	0	2	2	0	2	2	0	1	1	0	1	1	14	4	18
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	16	16	32
Chromosome Breaks	0	0	0	0	0	0	0	0	0	0	0	0	10	10	20
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total Aberrations	0	2	2	0	2	2	0	1	1	0	1	1	41	30	71
(excluding gaps)[b]			(1.0)			(1.0)			(0.5)			(0.5)			(71.0)
No. of cells with Aberr.	0	2	2	0	2	2	0	1	1	0	1	1	21	18	39[c]
(excluding gaps)[b]			(1.0)			(1.0)			(0.5)			(0.5)			(39.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2

[a] 1% Deionized Distilled Water.
[b] Values in parentheses are percentages.
[c] Significantly (alpha<0.01) different from the negative control.

Attachment 1 Page 2 of 2

TABLE 6A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D TIPA Positive Control: (0.5 µg/ml MMC)

	Neg. Control[a]			1250 µg/ml			2500 µg/ml			5000µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	0	5	5	14	1	15	1	0	1	0	4	4	2	4	6
Chromosome Gaps	1	2	3	1	1	2	4	1	5	4	0	4	5	2	7
Chromatid Breaks	1	1	2	6	0	6	2	3	5	4	4	8	5	6	11
Chromatid Exchanges	0	0	0	1	0	1	1	0	1	0	0	0	11	14	25
Chromosome Breaks	0	0	0	0	0	0	0	0	0	1	0	1	2	3	5
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Total Aberrations	1	1	2	8	0	8	3	3	6	6	4	10	18	23	41
(excluding gaps)[b]			(1.0)			(4.0)			(3.0)			(5.0)			(41.0)
No. of cells with Aberr.	1	1	2	7	0	7	2	3	5	5	4	9	16	21	37[c]
(excluding gaps)[b]			(1.0)			(3.5)			(2.5)			(4.5)			(37.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	1	0	1	0	0	0	0	0	0	4	5	9

[a] 1% Deionized Distilled Water
[b] Values in parentheses are percentages.
[c] Significantly (alpha<0.01) different from the negative control.

011942

DATA EVALUATION RECORD

2,4-D; triisopropylamine salt (2,4-D TIPA)

Study Type: 84-2; Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary Cells (CHO/HGPRT)

Work Assignment No. 1-16B (MRID 43327302)

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

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Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxcologist Jess Rowland Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head YIM Date 5/23/96
Review Section I, Toxicology Branch II(7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in Chinese hamster ovary cells.

Guideline Number: §84-2(2)

DP BARCODE: D207071 SUBMISSION: S472693

P.C. CODE: 030035 TOX. CHEM. NO.: 315AE

TEST MATERIAL): 2,4-Dichlorophenoxyacetic acid **triisopropanolamine** salt

SYNONYMS: 2,4-D **TIPA**

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-D triisopropanolamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay. Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID K-008866-018. 1/13/94. **MRID 43327302.**

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327302) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D TIPA salt formulation (70.9%) at concentrations of 800, 1,000, 1,250, 2,500, and 5,000 µg/mL with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at the limit dose of 5,000 µg/mL without S9 activation. **There was no evidence of mutagenic effect at any dose level with or without activation.** Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as **Acceptable** and satisfies the requirements for the 1991 Guideline 84-2(2) for *in vitro* mutagenicity (mammalian forward gene mutation) data.

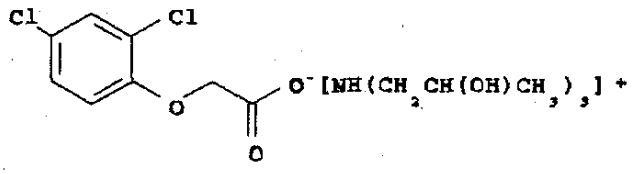
COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: 2,4-Dichlorophenoxyacetic acid triisopropanolamine (TIPA) salt
   Description: amber liquid
   Lot/Batch #: AGR 295711
   Purity: 70.9% a.i.
   Stability of compound: Not reported
   CAS #: 18584-79-7
   Structure:



   Solvent used: Water
   Other comments: None

2. Control Materials:
   Solvent/final concentration: culture medium
   Positive: Non-activation (concentrations, solvent):
   Ethylmethanesulfonate (EMS)/621 μg/mL in culture medium

   Activation (concentrations, solvent):
   20-Methylcholanthrene (20-MCA)/4 μg/mL in 1% DMSO/culture medium

3. Activation: S9 derived from

x Aroclor 1254	x induced	x rat x liver
__ phenobarbital	__ non-induced	__ mouse __ lung
__ none		__ hamster __ other
__ other		__ other

   Describe S9 mix composition (if purchased, give details): 10 mM $MgCl_2 \cdot 6\ H_2O$, 5 mM glucose-6-phosphate, 4 mM NADP, 10 mM $CaCl_2$, 30 mM KCl, and 50 mM sodium phosphate (pH 8.0)

4. Test Cells: Chinese hamster ovary (CHO) cells

Properly maintained? **Yes**
Periodically checked for Mycoplasma contamination? **Yes**
Periodically checked for karyotype stability? **Not** reported
Periodically "cleansed" against high spontaneous background? Not reported

Media: Ham's F-12 nutrient mix supplemented with 5% heat-activated fetal calf serum; 25 mM HEPES; Fungizone; penicillin G; and streptomycin sulfate.

5. Locus Examined:

__ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)
_____ trifluorothymidine (TFT)

_x_ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: _____ 8-azaguanine (8-AG)
10 µM 6-thioguanine (6-TG)

__ Na^+/K^+ ATPase
Selection agent: _____ ouabain
(give concentration)

__ other (locus and/or selection agent; give details):

6. Test compound concentrations used:

Non-activated conditions:
Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000 µg/mL µg/mL

Gene mutation Assay 1: 800, 1,000, 1,250, 2,500, and 5,000 µg/mL
Gene mutation Assay 2: 800, 1,000, 1,250, 2,500, and 5,000 µg/mL

Activated conditions:
Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000 µg/mL µg/mL

Gene mutation Assay 1: 800, 1,000, 1,250, 2,500, and 5,000 µg/mL
Gene mutation Assay 2: 800, 1,000, 1,250, 2,500, and 5,000 µg/mL

B. TEST PERFORMANCE

1. Cell treatment:
   a. Cells exposed to test compound, negative/solvent or positive controls for: 4 hours (non-activated) 4 hours (activated)

   b. After washing, cells cultured for 6-8 days (expression period) before cell selection:

   c. After expression, $2x10^5$ cells/dish (10 dishes/ group) were cultured for 7-9 days in selection medium to determine numbers of mutants and 200 cells/dish (3 dishes/group) were cultured for 7-9 days without selective agent to determine cloning efficiency.

2. Statistical Methods: The frequency of mutants per 10^6 cells was evaluated using weighted analysis of variance. Treated groups were compared to the vehicle control using a linear trend test and lack of fit test ($\alpha=0.05$). In the event of a significantly increasing trend or significant lack of fit, a Dunnett's t-test was conducted, and additional comparison of the positive control to the negative control was conducted using a linear contrast statement.

3. Evaluation Criteria: An assay was considered acceptable if the mutation frequency in the positive controls was significantly higher than the negative controls and if the negative controls were within reasonable limits of the laboratory historical control and literature values.

   The test chemical was considered positive if it induced a statistically significant, dose related, reproducible increase in mutation frequency compared to the vehicle control.

II. REPORTED RESULTS

A. Preliminary cytotoxicity assay: The cytotoxicity test (Table 1, study report page 20) was conducted with seven concentrations of 2,4-D TIPA salt ranging from 150 to 5,000 µg/mL with or without S9 activation. In the non-activated cultures, toxicity (~20% relative cell survival, RCS) was observed at the 5,000 µg/mL dose levels. In the presence of S9, RCS was 56% at the highest dose level. Based on these results, dose levels of 800-5,000 µg/mL were chosen for the test with and without S9 activation.

B. Mutagenicity assay: Analyses (HPLC) of the test material stock solutions from 800-5,000 µg/mL indicated that the actual concentrations were 96-104% of the target concentrations.

The mutagenicity assay results are presented in Tables 2A, 2B, 3A, and 3B, (study report pages 21-24). The mutation frequencies in cultures treated with the test material in the absence and presence of S9 were not significantly different from the concurrent negative controls and were within the laboratory historical background range of 0-2.6 (minimum) and 8.0-27.9 (maximum) TG' mutants per 10^6 cells over a 9-year period. In all assays, the positive control chemicals EMS (non-activated assay) and 20-MCA caused significant increases in mutation frequencies. Based on these results, the study author concluded that 2,4-D TIPA salt was not mutagenic in this *in vitro* mammalian cell test system.

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. The reviewer agrees with the study author, that the 2,4-D TIPA salt formulation did not induce mutation in this CHO/HGPRT mammalian forward gene mutation assay when tested to the limit dose of 5,000 µg/mL. The sensitivity of the test system to detect a mutagenic response was clearly demonstrated by the significant results obtained with the positive control substances, 621 µg/mL EMS in the non-activated system and 4 µg/mL 20-MCA in the S9 activated system. We conclude that the 2,4-D TIPA salt formulation is not mutagenic in this *in vitro* forward gene mutation system.

B. Study deficiencies: None

Table 1. Survival of CHO Cells Treated with the Test Chemical

Cell Line: CHO-K_1-BH_4 Passage 22 Test Chemical: 2,4-D TIPA

Treatment (µg/ml)	Without S-9 No. of Colonies is Dish 1	2	3	RCS (%)[a]	With S-9 No. of Colonies is Dish 1	2	3	RCS (%)[a]
Neg. Control[b]	124	134	116	100.0	150	139	134	100.0
150	126	119	112	95.5	144	133	134	97.2
300	130	113	142	102.9	155	147	131	102.4
600	171	262	195	167.9	122	132	164	98.8
800	129	151	121	107.2	159	124	149	102.1
1000	88	92	80	69.5	146	134	134	97.9
1250	104	130	125	96.0	152	117	132	94.8
2500	63	53	47	43.6	112	107	114	78.7
5000	28	25	22	20.1	87	73	77	56.0

[a] Relative Cell Survival (%) = $\frac{\text{Mean number of colonies/dish in the treated X 100}}{\text{Mean number of colonies/ dish in the negative control}}$

[b] 1% Water

Table 2A

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Absence of S-9

ASSAY 1

Cell Line: CHO-K$_1$-BH$_4$ Passage: 24

Test Chemical: 2,4-D TIPA Positive Control: 621 µg/ml EMS Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)				
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	124	129	126	107.4	0	0	0	0	0	0	0	0	0	0	0	147	140	173	76.7	0.0
Neg. Control	103	112	112	92.6	0	1	0	0	2	3	2	0	1	1	10	149	148	135	72.0	6.9
800	111	127	127	103.4	0	2	3	0	0	1	0	0	0	0	6	161	155	146	77.0	3.9
800	122	148	140	116.1	0	2	0	1	0	0	1	0	2	0	6	112	145	138	65.8	4.6
1000	93	83	92	75.9	0	0	0	1	1	0	0	0	0	0	2	140	166	137	73.8	1.4
1000	112	111	132	100.6	2	1	1	1	0	1	1	1	1	1	10	171	167	177	85.8	5.8
1250	121	114	118	100.0	0	0	0	0	0	1	1	0	0	2	4	164	166	151	80.2	2.5
1250	127	129	105	102.3	0	1	0	1	1	0	0	0	0	3	6	137	152	117	67.7	4.4
2500	103	119	105	92.6	0	0	0	0	0	0	0	0	0	1	1	165	138	160	77.2	0.6
2500	98	113	119	93.5	1	1	0	1	1	0	2	0	0	0	6	143	167	137	74.5	4.0
5000	45	36	38	33.7	1	0	0	0	0	0	0	1	0	1	3	111	109	117	56.2	2.7
5000	40	39	20	28.0	0	0	0	0	0	0	0	0	0	0	0	133	151	125	68.2	0.0
Pos. Control	67	76	49	54.4	19	14	23	20	6	9	15	22	19	7	154	81	70	65	36.0	213.9[d]
Pos. Control	68	75	67	59.5	23	21	21	37	23	25	11	19	17	22	219	5	77	73	25.8	423.9[d]

[a]Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b]TG^r = 6-Thioguanine resistant

[c]CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d]The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

Table 2B

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Absence of S-9

ASSAY 2

Cell Line: CHO-K_1 -BH_4 Passage: 28

Test Chemical: 2,4-D TIPA Positive Control: 621 µg/ml EMS Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)				
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	111	109	96	109.9	1	0	0	0	0	1	0	0	0	0	2	91	107	112	51.7	1.9
Neg. Control	94	82	83	90.1	0	0	0	0	0	1	0	0	0	0	1	156	122	107	64.2	0.8
800	84	72	103	90.1	0	0	0	0	1	0	0	0	1	1	3	108	107	91	51.0	2.9
800	73	76	98	85.9	1	2	1	1	0	3	2	1	1	1	13	118	117	120	59.2	11.0
1000	133	132	138	140.2	1	0	0	2	2	1	1	0	0	1	8	164	148	141	75.5	5.3
1000	129	129	130	135.0	2	0	1	0	0	1	0	0	2	0	6	94	82	141	52.8	5.7
1250	66	73	75	74.4	3	1	0	0	0	1	0	1	1	3	10	115	133	94	57.0	8.8
1250	129	130	158	145.0	0	2	2	0	1	0	0	1	1	1	8	142	115	113	61.7	6.5
2500	102	95	95	101.6	2	1	2	1	3	3	0	2	3	3	20	114	138	104	59.3	16.9
2500	71	81	93	85.2	0	0	0	0	1	1	1	0	3	1	7	117	163	131	68.5	5.1
5000	2	0	2	1.4					-[d]						-		-		-	-
5000	2	2	3	2.4					-[d]						-		-		-	-
Pos. Control	55	58	49	56.3	14	35	33	25	32	31	32	28	34	37	301	53	45	50	24.7	610.1[e]
Pos. Control	61	72	65	68.9	21	22	15	20	25	24	20	19	17	18	201		-[f]		-	NC[g]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] Data not available due to impaired proliferative capacity of the cells.

[e] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

[f] Data lost due to technical error.

[g] Not calculated.

Table 3A

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Presence of S-9

ASSAY 1

Cell Line: CHO-K_1 -BH_4 Passage: 24

Test Chemical: 2,4-D TIPA Positive Control: 4.0 µg/ml 20-MCA Negative Control: 1% WATER

	Toxicity Assay			Mutation Assay											Cloning Efficiency (CE)		
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	170	182	165	112.9	0	0	0	0	0	2	0	0	0	0	2	151	141	144	72.7	1.4
Neg. Control	125	141	133	87.1	3	2	0	0	0	2	4	1	1	1	14	178	172	174	87.3	8.0
800	152	162	167	105.0					-[d]						-	-			-	-
800	179	151	181	111.6	1	0	0	0	0	1	0	0	3	1	6	142	175	146	77.2	3.9
1000	122	104	98	70.7	0	1	0	1	0	0	0	0	0	0	2	172	140	164	79.3	1.3
1000	98	110	127	73.1	1	0	0	1	0	0	0	0	0	0	2	132	178	135	74.2	1.3
1250	159	176	185	113.5	1	2	1	3	2	1	0	4	1	1	16	139	158	142	73.2	10.9
1250	134	126	137	86.7	0	0	1	0	0	0	0	0	0	0	1	177	184	181	90.3	0.6
2500	144	133	128	88.4	0	0	0	0	0	0	2	0	0	0	2	168	176	172	86.0	1.2
2500	112	106	113	72.3	2	1	0	1	0	0	0	0	0	0	4	173	168	169	85.0	2.4
5000	54	52	39	31.7	1	0	0	0	1	1	1	0	2	0	6	190	169	155	85.7	3.5
5000	57	67	64	41.0	2	1	3	0	0	0	1	1	0	1	9	162	158	154	79.0	5.7
Pos. Control	141	142	144	93.2	13	16	14	12	17	9	16	18	14	15	144	141	151	156	74.7	96.4[e]
Pos. Control	128	156	125	89.3	26	18	17	10	19	18	14	20	13	11	166	140	124	151	69.2	120.0[e]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] Data lost due to lack of cells for plating.

[e] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

Table 3B

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Presence of S-9

ASSAY 2

Cell Line: CHO-K$_1$ -BH$_4$ Passage: 28

Test Chemical: 2,4-D TIPA Positive Control: 4.0 µg/ml 20-MCA Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay												Cloning Efficiency (CE)			
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]											Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	104	103	103	86.2	0	2	1	0	0	0	0	1	0	0		4	127	128	133	64.7	3.1
Neg. Control	147	126	136	113.8	1	0	1	3	1	0	0	0	0	0		6	98	114	116	54.7	5.5
800	101	106	123	91.8	3	2	0	1	2	1	0	4	0	1		14	152	124	118	65.7	10.7
800	97	97	104	82.9	1	3	2	0	0	1	0	0	0	0		7	129	129	119	62.8	5.6
1000	106	120	133	99.9	0	0	1	1	1	1	0	0	1	1		6	89	122	107	53.0	5.7
1000	110	86	119	87.6	0	1	1	0	1	0	2	1	0	1		7	130	120	95	57.5	6.1
1250	133	142	142	116.0					-[d]							-		-		-	-
1250	105	80	97	78.4	0	1	0	0	0	0	1	1	0	0		3	122	107	101	55.0	2.7
2500	87	89	78	70.7	3	3	2	0	1	2	2	3	0	3		19	121	144	113	63.0	15.1
2500	92	114	110	87.9	0	0	0	0	0	0	0	0	0	0		0	111	127	116	59.0	0.0
5000	49	40	61	41.7	1	0	0	1	0	1	1	0	4	1		9	111	84	97	48.7	9.2
5000	53	27	50	36.2	2	1	0	0	0	0	0	0	0	1		4	150	131	127	68.0	2.9
Pos. Control	82	82	94	71.8	21	20	26	16	17	28	15	16	19	17		195	120	135	123	63.0	154.8[e]
Pos. Control	95	86	106	79.8	16	19	20	7	15	12	17	18	27	23		174	96	109	150	59.2	147.0[e]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] Data lost due to lack of cells for plating

[e] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

011942

DATA EVALUATION RECORD

2,4-D; isopropylamine salt (2,4-D IPA)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16C (MRID 43327303)

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
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Prepared by

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Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist Jess Rowland Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head J Mh Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in rat lymphocytes

OPP Guideline Number: §84-2(2)

DP BARCODE: D207071 SUBMISSION: S472693

P.C. CODE: 030025 TOX. CHEM. NO.: 315U

TEST MATERIAL: 2,4-dichlorophenoxyacetic acid **isopropylamine** salt;

SYNONYMS: 2,4-D **IPA**

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID M-004725-016, Study dates: 5/19/93-5/27/94. **MRID No. 43327303.** Unpublished.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327303), rat lymphocyte cultures were exposed to a 2,4-D IPA formulation (50.2% a.i.), in deionized distilled water for 4 hours at concentrations of 96, 192, 384, 767, 1,534, 3,068, and 6,137 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D IPA was tested to 6,137 µg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 28-43% of negative control without S9 activation or 48-53% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. The statistically significant increase in the proportion of aberrant cells at the highest concentration over the negative control value was determined not to be biologically significant since the background aberration frequency for rat lymphocytes can range from 0-5.5% and statistical significance was seen only because the concurrent solvent control value was 0%. **Therefore it was concluded that 2,4-D IPA at upto 3000 µg/mL was not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.**

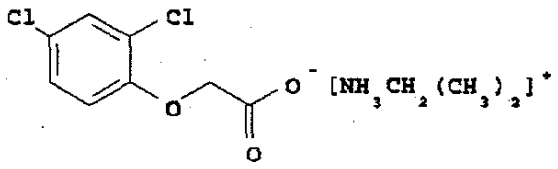
This study is classified as **Acceptable** and does satisfy the requirements for the 1991 Guideline 84-2(2) for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: 2,4-D IPA
Description: amber liquid
Lot/Batch #: AGR 276461
Purity: The test material is a formulation intermediate containing 50.2% 2,4-D IPA salt. The acid (2,4-D) equivalent = 40.9%
Stability of compound: Not addressed
CAS #: 5742-17-6
Structure:



Solvent used: Deionized distilled water
Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:
Negative: Solvent control
Solvent/final concentration: Deionized distilled water/1%
Positive: Nonactivation: Mitomycin C (0.5 µg/mL)
Activation: Cyclophosphamide (6 µg/mL)

3. Activation: S9 derived from

_X_ Aroclor 1254	_X_ induced	_X_ rat	_X_ liver
___ phenobarbital	___ non-induced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other			___ other

S9 purchased from Sitek Research Laboratories, Rockville, Maryland
S9 mix composition: S9 fraction (10% v/v), $MgCl_2$ (10mM), Na_2HPO_4 buffer (50mM), pH 8.0, glucose-6-phosphate (5mM), NADP (4mM), $CaCl_2$ (10mM), KCl (30mM)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

Nonactivated conditions:
Assay 1*: 4 hour treatment (96, 192, 384, 767, 1,534, 3,068, 6,137 µg/mL), harvest at 24 hours

Assay 2: 4 hour treatment (750, 1,500, 3,000 µg/mL), harvest at 24 and 48 hours

Activated conditions:
Assay 1*: 4 hour treatment (96, 192, 384, 767, 1,534, 3,068, 6,137 µg/mL), harvest at 24 hours

Assay 2: 4 hour treatment (375, 750, 1,500 µg/mL), harvest at 24 and 48 hours

*The laboratory indicated that the concentrations used were approximately 23% higher than targeted concentrations because the purity of the test material (40.9%), as originally provided by the sponsor, was actually the acid equivalent of 2,4-D. The purity of the salt was later reported to be 50.2%.

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20 µg/mL).

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? N/A

Cell line or strain periodically checked for karyotype stability? N/A

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5 mg/mL, whichever is lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected initially for subsequent metaphase analysis at 48-hour harvest).

2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to the test chemical or positive control for a selected time interval, then the chemical was removed and the cells continued in culture until time of harvest

a. Cell treatment:
Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)

Assay 1: 4 hr exposure, -S9, harvest at 24 hrs
4 hr exposure, +S9, harvest at 24 hrs

Assay 2: 4 hr exposure, -S9, harvest at 24 hrs
4 hr exposure, +S9, harvest at 24 hrs
4 hr exposure, -S9, harvest at 48 hrs
4 hr exposure, +S9, harvest at 48 hrs

b. Spindle inhibition
Inhibitor used/concentration: Colcemid (0.2 µg/mL)
Administration time: 3 hours (before cell harvest)

c. Cell harvest:
Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).

d. Details of slide preparation:
Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.

e. Metaphase analysis
No. of cells examined per dose: 200
Solvent control: 200
Positive control: 100

Scored for structural: **Yes**
Scored for numerical: **No**
Coded prior to analysis: **Yes**

f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.

g. Statistical analysis: Data evaluated for statistical significance at α=0.01, using Chi-square statistics from two way contingency table.

II. REPORTED RESULTS

Analysis of dose formulations by HPLC indicated that concentrations were between 115 and 129% of target concentrations for Assay 1 and between 100 and 107% for Assay 2.

A. Preliminary cytotoxicity assay:
Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, both with and without S9 activation, the 6,137 µg/mL concentration reduced the mitotic index to 0%. At 3,068 µg/mL without S9 activation the mitotic index was 43% of the negative control value. With S9 activation, only one of two cultures survived treatment at 3068 µg/mL. At 1,534 µg/mL, the mitotic index was 53% of the negative control value.

In Assay 2, without S9 activation, 3,000 µg/mL reduced the mitotic index to 28% of the negative control value 24 hours after treatment. By 48 hours the mitotic index was still 33% of the negative control. With S9 activation, 1,500 µg/mL reduced the mitotic index to 48% of the negative control value 24 hours after treatment. By 48 hours the mitotic index returned to 90% of the negative control.

B. Cytogenetic assay: Results are presented in Attachments 1-3 (study report pages 23, 24, 27-30).

In Assay 1, cultures treated with 767, 1,534, and 3,068 µg/mL of the test compound without S9 activation, or 384, 767, and 1,534 µg/mL of the test compound with S9 activation, were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1).

2,4-D IPA caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The aberration frequencies of the treated cultures in the presence of S9 activation were outside the historical range of the laboratory; however, in the confirmatory study (Assay 2) they were within the historical range. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory assay 2, cultures treated with 750, 1,500, and 3,000 µg/mL of the test compound without S9 activation, or 375, 750, and 1,500 µg/mL of the test compound with S9 activation were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 2). The 2,4-D IPA formulation caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds. At the 48-hour harvest only cultures from the negative control and highest treatment level were initially analyzed for chromosomal aberration frequencies. The number of aberrant cells in cultures treated at 3,000 µg/mL without S9 activation was significantly higher than the negative control value (6% vs 0%). Subsequent analysis of the two lower concentrations did not show significant increases over control values (Attachment 3).

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. The study authors suggest that the statistically identified increase in aberrant cells at the 48-hour harvest in cultures treated with 3,000 µg/mL without S9 activation is due to the occurrence of 0% aberrant cells in the concurrent negative controls. They further state that the incidence of aberrant cells among negative control cultures of this study ranged from 0-5.5% and the laboratory historical negative control values ranged from 0-6.5%. The reviewers concur with the study authors and conclude that 2,4-D IPA at upto 3000 µg/mL is not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.

B. Study deficiencies None.

ATTACHMENTS

TABLE 4A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D IPA Negative Control: 1% Water Positive Control: (0.5 µg/ml MMC)

	Neg. Control			*767 µg/ml*			*1534 µg/ml*			*3068 µg/ml*			*Positive control*		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	5	2	7	3	3	6	6	6	12	6	7	13	10	11	21
Chromosome Gaps	1	0	1	2	2	4	1	1	2	1	1	2	3	0	3
Chromatid Breaks	3	1	4	6	3	9	6	4	10	6	2	8	8	22	30
Chromatid Exchanges	0	0	0	0	0	0	0	2	2	2	0	2	20	26	46
Chromosome Breaks	1	1	2	1	0	1	1	3	4	2	1	3	2	5	7
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Total Aberrations	4	2	6	7	3	10	7	9	16	11	3	14	31	53	84
(excluding gaps)[a]			(3.0)			(5.0)			(8.0)			(7.0)			(84.0)
No. of cells with Aberr.	4	2	6	7	3	10	7	7	14	9	2	11	33	37	70[b]
(excluding gaps)[a]			(3.0)			(5.0)			(7.0)			(5.5)			(70.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	13	8	21

[a]Values in parentheses are percentages.
[b]Significantly (alpha<0.01) different from the negative control.

Attachment 1 Page 1 of 2

TABLE 4B

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D IPA Negative Control: 1% Water Positive Control: (6 µg/ml CP)

	Neg. Control			384 µg/ml			767 µg/ml			1534 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	8	4	12	7	2	9	6	5	11	6	5	11	1	8	9
Chromosome Gaps	1	1	2	0	0	0	1	0	1	2	3	5	5	0	5
Chromatid Breaks	4	6	10	5	8	13	6	8	14	7	9	16	17	25	42
Chromatid Exchanges	0	0	0	0	0	0	0	3	3	6	1	7	3	14	17
Chromosome Breaks	0	2	2	3	0	3	2	0	2	2	5	7	6	5	11
Chromosome Exchanges	0	0	0	1	0	1	0	0	0	1	0	1	0	1	1
Total Aberrations	4	8	12	9	8	17	8	11	19	16	15	31	26	46	72
(excluding gaps)[a]			(6.0)			(8.5)			(9.5)			(15.5)			(72.0)
No. of cells with Aberr.	4	7	11	8	8	16	7	8	15	11	14	25	23	36	59[b]
(excluding gaps)[a]			(5.5)			(8.0)			(7.5)			(12.5)			(59.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	3	3	6	9	15

[a]Values in parentheses are percentages.
[b]Significantly (alpha<0.01) different from the negative control.

Attachment 1 Page 2 of 2

TABLE 6A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D IPA *Negative Control:* 1% Water *Positive Control:* (0.5 µg/ml MMC)

	Neg. Control			750 µg/ml			1500 µg/ml			3000 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	1	2	3	1	4	5	1	1	2	5	3	8	3	5	8
Chromosome Gaps	1	1	2	2	0	2	1	3	4	4	3	7	3	2	5
Chromatid Breaks	3	2	5	3	1	4	0	1	1	2	2	4	18	15	33
Chromatid Exchanges	0	0	0	0	0	0	2	3	5	3	0	3	18	25	43
Chromosome Breaks	0	0	0	0	0	0	0	1	1	0	2	2	1	6	7
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Total Aberrations	3	2	5	3	1	4	2	5	7	5	4	9	38	46	84
(excluding gaps)[a]			(2.5)			(2.0)			(3.5)			(4.5)			(84.0)
No. of cells with Aberr.	3	2	5	3	1	4	1	5	6	2	4	6	24	28	52[b]
(excluding gaps)[a]			(2.5)			(2.0)			(3.0)			(3.0)			(32.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Values in parentheses are percentages.
[b]Significantly (alpha<0.01) different from the negative control.

TABLE 6C

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D IPA Negative Control: 1% Water Positive Control: (6 µg/ml CP)

	Neg. Control			375 µg/ml			750 µg/ml			1500 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	1	2	3	0	1	1	2	0	2	0	0	0	2	1	3
Chromosome Gaps	0	0	0	1	0	1	0	1	1	2	2	4	4	3	7
Chromatid Breaks	0	3	3	0	0	0	3	1	4	2	0	2	8	5	13
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	3	3	4	3	7
Chromosome Breaks	0	1	1	1	0	1	2	0	2	1	1	2	11	7	18
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Total Aberrations	0	4	4	1	0	1	5	1	6	3	4	7	23	16	39
(excluding gaps)[a]			(2.0)			(0.5)			(3.0)			(3.5)			(39.0)
No. of cells with Aberr.	0	4	4	1	0	1	5	1	6	3	1	4	18	14	32[b]
(excluding gaps)[a]			(2.0)			(0.5)			(3.0)			(2.0)			(32.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	4	2	6

[a]Values in parentheses are percentages.
[b]Significantly (alpha<0.01) different from the negative control.

Attachment 2 Page 2 2

TABLE 6B

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 48 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D IPA Negative Control: 1% Water

	Neg. Control			750 µg/ml			1500 µg/ml			3000 µg/ml		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200
Chromatid Gaps	0	1	1	0	1	1	2	1	3	4	1	5
Chromosome Gaps	0	1	1	0	0	0	1	2	3	1	5	6
Chromatid Breaks	0	0	0	1	2	3	1	1	2	4	6	10
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	1	1
Chromosome Breaks	0	0	0	0	0	0	1	0	1	0	0	0
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0
Total Aberrations	0	0	0	1	2	3	2	1	3	4	7	11
(excluding gaps)[a]			(0.0)			(1.5)			(1.5)			(5.5)
No. of cells with Aberr.	0	0	0	1	2	3	2	1	3	5	7	12[b]
(excluding gaps)[a]			(0.0)			(1.5)			(1.5)			(6.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	1	0	1

[a]Values in parentheses are percentages.
[b]Significantly (alpha<0.01) different from the negative control.

Attachment 3 Page 1 of 2

TABLE 6D

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 48 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D IPA Negative Control: 1% Water

	Neg. Control			375 µg/ml			750 µg/ml			1500 µg/ml		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200
Chromatid Gaps	1	2	3	4	2	6	0	2	2	3	3	6
Chromosome Gaps	0	1	1	0	0	0	1	1	2	0	2	2
Chromatid Breaks	0	1	1	0	0	0	0	0	0	2	2	4
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	0	0
Chromosome Breaks	0	0	0	0	0	0	0	0	0	3	0	3
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0
Total Aberrations	0	1	1	0	0	0	0	0	0	5	2	7
(excluding gaps)[a]			(0.5)			(0.0)			(0.0)			(3.5)
No. of cells with Aberr.	0	1	1	0	0	0	0	0	0	4	2	6
(excluding gaps)[a]			(0.5)			(0.0)			(0.0)			(3.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0

[a]Values in parentheses are percentages.

Table 5A

MITOTIC INDICIES (M.I.) OF RAT LYMPHOCYTE CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF THE TEST MATERIAL (2, 4-D IPA) IN THE ABSENCE OF S-9

ASSAY 2

	%M.I. Harvested 24 h After Treatment			% M.I. Harvested 48 h After Treatment		
Dose µg/ml	Replicate A	Replicate B	Average A+B	Replicate A	Replicate B	Average A+B
Negative control[a]	8.7	7.8	8.3	7.8	4.9	6.4
750	7.4	6.7	7.1	4.7	5.0	4.9
1500	5.5	6.4	6.0	5.1	5.6	5.4
3000	3.0	1.6	2.3	2.3	1.9	2.1
Positive control[b]	2.9	2.0	2.5	ND[c]	ND	ND

[a]1% Water
[b]MMC (0.5 µg/ml)
[c]ND = Not Done

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DATA EVALUATION RECORD

2,4-D; isopropylamine salt (2,4-D IPA)

Study Type: 84-2; Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary Cells (CHO/HGPRT)

Work Assignment No. 1-16D (MRID 43327304)

Prepared for

Health Effects Division
Office of Pesticide Programs
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1921 Jefferson Davis Highway
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Date: 4/15/96

Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist. Jess Rowland Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head Y.M.I. Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in Chinese hamster ovary cells.

Guideline Number: §84-2(2)

DP BARCODE: D206005 SUBMISSION: S472693

P.C. CODE: 030025 TOX. CHEM. NO.: 315U

TEST MATERIAL: 2,4-Dichlorophenoxyacetic acid **isopropylamine** salt

SYNONYMS: 2,4-D **IPA**

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4- Dichlorophenoxyacetic acid isopropylamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward mutation Assay. Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID M-004725-017. May 27, 1994. **MRID 43327304.**

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327304) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D IPA salt formulation (50.2%) at concentrations of 500, 1,000, 1,500, 2,000, and 3,000 µg/mL with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at a dose of 2,500 µg/mL with and without S9 activation. **There was no evidence of a mutagenic effect at any dose level with or without activation.** Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

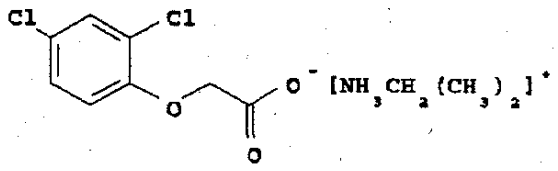
This study is classified as **acceptable** and satisfies the requirements for FIFRA Test Guideline 84-2 for *in vitro* mutagenicity (mammalian forward gene mutation) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: 2,4-Dichlorophenoxyacetic acid isopropylamine (IPA) salt
   Description: amber liquid
   Lot/Batch #: AGR 276461
   Purity: 50.2% a.i.
   Stability of compound: Not reported
   CAS #: 5742-17-6
   Structure:



   Solvent used: Water
   Other comments: None

2. Control Materials:
   Solvent/final concentration: culture medium
   Positive: Non-activation (concentrations, solvent):
   Ethylmethanesulfonate (EMS)/621 μg/mL in culture medium

   Activation (concentrations, solvent):
   20-Methylcholanthrene (20-MCA)/4 μg/mL in 1% DMSO/culture medium

3. Activation: S9 derived from

x Aroclor 1254	x induced	x rat	x liver
__ phenobarbital	__ non-induced	__ mouse	__ lung
__ none		__ hamster	__ other
__ other		__ other	

   Describe S9 mix composition (if purchased, give details): 10 mM $MgCl_2 \cdot 6\ H_2O$, 5 mM glucose-6-phosphate, 4 mM NADP, 10 mM $CaCl_2$, 30 mM KCl, and 50 mM sodium phosphate (pH 8.0)

4. Test Cells: Chinese hamster ovary (CHO) cells

Properly maintained? **Yes**
Periodically checked for Mycoplasma contamination? **Yes**
Periodically checked for karyotype stability? **Not** reported
Periodically "cleansed" against high spontaneous background? Not reported

Media: Ham's F-12 nutrient mix supplemented with 5% heat-activated fetal calf serum; 25 mM HEPES; Fungizone; penicillin G; and streptomycin sulfate.

5. Locus Examined:

__ thymidine kinase (TK)
Selection agent: _____ bromodeoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)
_____ trifluorothymidine (TFT)

x hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: _____ 8-azaguanine (8-AG)
10 µM ___ 6-thioguanine (6-TG)

__ Na^+/K^+ ATPase
Selection agent: _____ ouabain
(give concentration)

__ other (locus and/or selection agent; give details):

6. Test compound concentrations used:

Non-activated conditions:
Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000 µg/mL µg/mL

Gene mutation Assay: 500, 1,000, 1,500, 2,000, and 3,000 µg/mL

Activated conditions:
Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000 µg/mL

Gene mutation Assay: 500, 1,000, 1,500, 2,000, and 3,000 µg/mL

B. TEST PERFORMANCE

1. Cell treatment:

a. Cells exposed to test compound, negative/solvent or positive controls for: 4 hours (non-activated) 4 hours (activated)

b. After washing, cells cultured for 6-8 days (expression period) before cell selection:

c. After expression, $2x10^5$ cells/dish (10 dishes/ group) were cultured for 7-9 days in selection medium to determine numbers of mutants and 200 cells/dish (3 dishes/group) were cultured for 7-9 days without selective agent to determine cloning efficiency.

2. Statistical Methods: The frequency of mutants per 10^6 cells was evaluated using weighted analysis of variance. Treated groups were compared to the vehicle control using a linear trend test and lack of fit test ($\alpha=0.05$). In the event of a significantly increasing trend or significant lack of fit, a Dunnett's t-test was conducted, and additional comparison of the positive control to the negative control was conducted using a linear contrast statement.

3. Evaluation Criteria: An assay was considered acceptable if the mutation frequency in the positive controls was significantly higher than the negative controls and if the negative controls were within reasonable limits of the laboratory historical control and literature values.

The test chemical was considered positive if it induced a statistically significant, dose related, reproducible increase in mutation frequency compared to the vehicle control.

II. REPORTED RESULTS

A. Preliminary cytotoxicity assay: The cytotoxicity test was conducted with seven concentrations of a 2,4-D IPA salt formulation ranging from 150 to 5,000 µg/mL with or without S9 activation (Table 1, study report page 20). The report stated that the actual concentrations were 23% higher than the targets owing to initial information from the sponsor that the purity of the test article was 40.9% a.i. instead of 50.2%. In the non-activated cultures, toxicity (1.6% relative cell survival, RCS) was observed at the 2,500 µg/mL dose level. In the presence of S9, RCS was 0.8% at the 5,000 µg/mL dose level and 5.4% at 2,500 µg/mL. Based on these results, dose levels of 500-3,000 µg/mL were chosen for the mutagenicity test with and without S9 activation.

B. Mutagenicity assay: Analyses (HPLC) of the test material stock solutions from 500-3,000 µg/mL indicated that the actual concentrations were 84-107% of the target concentrations.

The mutagenicity assay results are presented in Tables 2A, 2B, 3A, and 3B (study report pages 21-24). The mutation frequencies in cultures treated with the test material in the absence and presence of S9 were not significantly different from the concurrent negative controls and were within the laboratory historical background range of 0-2.6 (minimum) and 8.0-27.9 (maximum) TG' mutants per 10^6 cells over a 9-year period. In all assays, the positive control chemicals EMS (non-activated assay) and 20-MCA caused significant increases in mutation frequencies. Based on these results, the study author concluded that 2,4-D IPA salt was not mutagenic in this *in vitro* mammalian cell test system.

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. The reviewer agrees with the study author, that the 2,4-D IPA salt formulation did not induce mutation in this CHO/HGPRT mammalian forward gene mutation assay when tested to the limit dose of 5,000 µg/mL. The sensitivity of the test system to detect a mutagenic response was clearly demonstrated by the significant results obtained with the positive control substances, 621 µg/mL EMS in the non-activated system and 4 µg/mL 20-MCA in the S9 activated system. We conclude that 2,4-D IPA salt is not mutagenic in this *in vitro* forward gene mutation system.

B. Study deficiencies: None

Table 1. Survival of CHO Cells Treated with the Test Chemical

Cell Line: CHO-K_1-BH_4 Passage 23 Test Chemical: 2,4-D IPA

Treatment (µg/ml)	Without S-9 No. of Colonies is Dish 1	2	3	RCS (%)[a]	With S-9 No. of Colonies is Dish 1	2	3	RCS (%)[a]
Neg. Control[b]	146	144	143	100.0	163	155	162	100.0
150	132	178	140	103.9	183	136	176	103.1
300	175	197	182	127.9	134	134	134	83.8
600	177	183	204	130.3	205	192	200	124.4
800	155	160	157	109.0	108	100	88	61.7
1000	160	162	138	106.2	102	129	118	72.7
1250	44	36	33	26.1	118	93	82	61.0
2500	1	2	4	1.6	9	12	5	5.4
5000	0	0	0	0.0	1	1	2	0.8

[a] Relative Cell Survival (%) = $\frac{\text{Mean number of colonies/dish in the treated X 100}}{\text{Mean number of colonies/ dish in the negative control}}$

[b] 1% Water

Table 2A

Results of the Gene Mutation Assay In CHO Cells treated with Test Chemical in the Absence of S-9

ASSAY 1

Cell Line: CHO-K_1-BH_4 Passage: 24

Test Chemical: 2,4-D IPA Positive Control: 621 µg/ml EMS Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)				
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	159	161	143	98.6	0	0	1	0	0	0	0	1	0	1	2	121	123	157	66.8	1.5
Neg. Control	165	166	145	101.4	0	0	0	0	0	0	1	0	0	1	2	125	106	102	55.5	1.8
500	157	134	179	100.1	1	0	0	0	0	0	1	0	0	1	3	122	102	107	55.2	2.7
500	156	138	149	94.4	0	0	0	0	0	0	0	0	0	0	0	131	167	134	72.0	0.0
1000	133	139	133	86.3	0	2	0	0	0	2	0	2	1	0	7	173	163	134	78.3	4.5
1000	176	168	139	102.9	0	0	1	1	1	2	0	1	0	0	6	126	142	141	68.2	4.4
1500	135	137	133	86.3	0	0	1	1	0	0	1	1	1	1	6	155	186	151	82.0	3.7
1500	131	133	146	87.3	0	0	0	0	1	0	1	1	1	0	4	146	168	167	80.2	2.5
2000	110	119	118	73.9	1	0	0	1	3	0	0	0	0	0	5	126	136	109	61.8	4.0
2000	117	113	113	73.1	1	0	0	0	0	0	0	1	0	1	3	152	132	133	69.5	2.2
3000	77	67	76	46.9	0	2	1	1	0	3	1	0	1	1	10	158	150	177	80.8	6.2
3000	72	58	73	43.2	0	0	0	0	0	0	0	0	0	0	0	160	149	129	73.0	0.0
Pos. Control	60	75	77	45.2	7	8	8	16	13	11	9	9	8	6	95	57	62	62	30.2	157.5[d]
Pos. Control	54	63	49	35.4	13	20	10	14	17	14	16	9	28	23	164	64	61	61	31.0	264.5[d]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\textit{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

Table 2B

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Absence of S-9

ASSAY 2

Cell Line: CHO-K_1-BH_4 Passage: 24

Test Chemical: 2,4-D IPA Positive Control: 621 µg/ml EMS Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)				
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	88	116	106	95.4	0	0	0	0	1	0	0	0	0	0	1	114	103	113	55.0	0.9
Neg. Control	106	113	121	104.6	0	0	0	0	0	0	0	0	0	0	0	157	142	138	72.8	0.0
500	135	143	139	128.3	2	0	0	0	0	0	0	0	0	0	2	145	153	133	71.8	1.4
500	138	146	164	137.8	0	0	1	0	0	0	3	1	0	2	7	125	126	112	60.5	5.8
1000	141	184	148	139.4	0	0	3	1	0	4	3	0	1	0	12	143	125	130	66.3	9.0
1000	149	148	140	134.5	1	0	1	0	1	0	2	1	0	4	10	102	114	113	54.8	9.1
1500	152	113	143	125.5	1	0	0	1	2	1	1	0	0	1	7	137	118	129	64.0	5.5
1500	133	149	171	139.4	3	1	1	1	3	2	1	2	0	2	16	154	132	127	68.8	11.6
2000	102	85	87	84.3					-[d]						-	-			-	-
2000	102	97	118	97.5	0	0	2	2	1	1	0	1	1	2	10	118	104	76	49.7	10.1
3000	41	37	41	36.6					-[e]						-	-			-	-
3000	40	46	46	40.6	1	0	4	0	1	0	9	2	0	1	18	119	125	124	61.3	14.7
Pos. Control	60	59	65	56.6	20	17	19	16	13	20	7	19	14	18	163	56	55	53	27.3	298.2[f]
Pos. Control	46	59	74	55.1	14	28	24	23	15	24	17	20	18	17	200	54	69	48	28.5	350.9[f]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] Data lost due to technical error

[e] Data not available due to impaired proliferative capacity of the cells

[f] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05)

Table 3A

Results of the Gene Mutation Assay In CHO Cells treated with Test Chemical In the Presence of S-9

ASSAY 1

Cell Line: CHO-K_1-BH_4 Passage: 22

Test Chemical: 2,4-D IPA Positive Control: 4.0 µg/ml 20-MCA Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)			
Treatment (µg/ml)	No. Colonies In Individual Dishes			RCS (%)[a]	TG^r Colonies In Individual Dishes[b]										Total	No of Colonies In Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	147	119	151	110.8	1	0	0	0	1	1	0	1	0	0	4	175	165	169	84.8	2.4
Neg. Control	104	112	120	89.2	0	1	0	0	0	0	0	0	0	0	1	133	138	122	65.5	0.8
500	122	105	121	92.4	0	0	1	1	0	1	0	1	0	0	4	115	149	123	64.5	3.1
500	96	105	135	89.2	0	0	0	0	0	1	1	1	1	0	4	190	190	205	97.5	2.1
1000	106	113	120	90.0	1	2	2	0	0	1	1	0	1	0	8	184	176	176	89.3	4.5
1000	126	119	121	97.2	0	1	2	0	1	0	1	1	2	3	11	158	162	157	79.5	6.9
1500	110	113	110	88.4	1	1	1	0	1	0	0	0	0	1	5	149	140	142	71.8	3.5
1500	121	147	114	101.5	2	2	2	0	0	1	0	1	0	1	9	139	124	153	69.3	6.5
2000	149	133	117	106.0	1	2	0	0	0	0	0	1	0	3	7	149	147	131	71.2	4.9
2000	125	121	95	90.6	1	2	2	1	2	0	1	1	2	1	13	144	135	123	67.0	9.7
3000	79	82	83	64.8	1	1	2	1	2	2	2	1	0	1	13	177	194	165	89.3	7.3
3000	93	83	83	68.8	1	0	0	0	1	0	2	0	2	0	6	200	145	158	83.8	3.6
Pos. Control	97	91	97	75.7	16	18	19	19	17	8	8	18	11	15	149	166	162	167	82.5	90.3[d]
Pos. Control	102	81	69	66.9	21	13	25	16	12	24	25	16	13	16	181	167	171	170	84.7	106.9[d]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated x 100}}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish x 100}}{\text{No. of cells seeded/dish}}$

[d] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05).

Table 3B

Results of the Gene Mutation Assay in CHO Cells treated with Test Chemical in the Presence of S-9

ASSAY 2

Cell Line: CHO-K_1-BH_4 Passage: 24

Test Chemical: 2,4-D IPA Positive Control: 4.0 µg/ml 20-MCA Negative Control: 1% WATER

	Toxicity Assay				Mutation Assay											Cloning Efficiency (CE)			
Treatment (µg/ml)	No. Colonies in Individual Dishes			RCS (%)[a]	TG^r Colonies in Individual Dishes[b]										Total	No of Colonies in Individual Dishes			CE (%)[c]	TG^r Mutants per 10^6 Clonable Cells
Neg. Control	124	130	113	99.3	0	0	0	0	0	0	0	0	0	0	0	162	168	126	76.0	0.0
Neg. Control	123	109	140	100.7	1	2	2	0	0	1	1	0	0	1	8	131	131	162	70.7	5.7
500	102	104	119	88.0	3	3	1	1	0	3	1	1	1	1	15	144	121	132	66.2	11.3
500	121	115	121	96.6	0	0	2	1	2	0	0	0	0	2	7	161	131	124	69.3	5.0
1000	105	106	89	81.2	0	1	0	0	2	0	0	1	1	1	6	110	117	114	56.8	5.3
1000	112	80	97	78.2	1	1	0	0	1	0	1	1	2	0	7	123	130	147	66.7	5.3
1500	104	95	107	82.8	0	2	2	0	2	1	0	0	0	1	8	129	127	140	66.0	6.1
1500	102	90	97	78.2	0	0	1	0	0	0	0	0	0	1	2	138	132	137	67.8	1.5
2000	102	125	79	82.8	1	1	1	0	0	0	2	2	1	1	9	138	115	150	67.2	6.7
2000	129	119	117	98.8	-[d]										-	-			-	-
3000	72	76	80	61.7	3	3	4	0	1	0	3	1	1	2	18	138	142	130	68.3	13.2
3000	17	26	31	20.0	0	1	0	0	0	0	0	0	0	3	4	127	109	95	55.2	3.6
Pos. Control	124	122	126	100.7	7	15	12	26	11	12	12	15	14	18	142	105	136	136	62.8	113.0[e]
Pos. Control	110	98	112	86.8	13	21	14	18	21	15	13	12	22	22	171	138	145	118	66.8	127.9[e]

[a] Relative cell survival (%) = $\frac{\text{Mean number of colonies/ dish in the treated} \times 100}{\text{Mean number of colonies/ dish in the negative control (avg. of replicates)}}$

[b] TG^r = 6-Thioguanine resistant

[c] CE (%) = $\frac{\text{Mean number of colonies/dish} \times 100}{\text{No. of cells seeded/dish}}$

[d] Data lost due to lack of cells for plating

[e] The frequency of TG^r mutants is significantly higher than the concurrent negative control value (alpha=0.05)

011942

DATA EVALUATION RECORD

2,4-D; butoxyethyl ester (2,4-D BEE)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16E (MRID 43327305)

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
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Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist Jess Rowland Date 5/22/96
Review Section I, Toxicology Branch II (7509C)

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head Y.M.I. Date 5/23/96
Review Section I, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in rat lymphocytes

Guideline Number: §84-2(2)

DP BARCODE: D207071 SUBMISSION: S472693

P.C. CODE: 030053 TOX. CHEM. NO.: 315AE

TEST MATERIAL: 2,4-dichlorophenoxyacetic acid **butoxyethyl ester**

SYNONYMS: 2,4-D **BEE**

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences, Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-007722-022, Study dates: 5/19/93-5/27/94. **MRID No. 43327305.** Unpublished.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327305), rat lymphocyte cultures were exposed to 2,4-D BEE (94.6% a.i.), in dimethyl sulfoxide for 4 hours at concentrations of 87.5, 175, 350, 700, and 1,400 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D BEE was tested to the limit of solubility, 1.4 mg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 31-80% of negative control without S9 activation or 61-67% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response.

There were no statistically significant increases in the proportion of aberrant cells over negative control values. However, cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis. In addition, the highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity, while this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay.

Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of metabolic activation, based on the results of this study, cannot be made at this time. It is critical that this chemical be evaluated up to a reproducible cytotoxic level. **It is concluded that a new study should be conducted.**

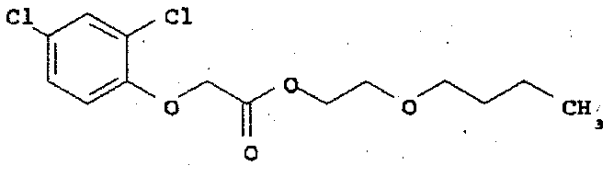
This study is classified as **unacceptable** and does not satisfy the requirements for the 1991 Guideline, 84-2 for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: 2,4-D BEE
Description: amber liquid
Lot/Batch #: AGR 276426
Purity: 94.6%
Stability of compound: Not addressed
CAS #: 1929-73-3
Structure:



Solvent used: Dimethyl sulfoxide (DMSO)
Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:
Negative: Solvent control
Solvent/final concentration: Dimethyl sulfoxide/1%
Positive: Nonactivation: Mitomycin C (MMC, 0.5 µg/mL)
Activation: Cyclophosphamide (CP, 6 µg/mL)
Other comments: DMSO not used as solvent for positive controls. MMC and CP dissolved directly in treatment medium

3. Activation: S9 derived from

_X_ Aroclor 1254	_X_ induced	_X_ rat _X_ liver
___ phenobarbital	___ non-induced	___ mouse ___ lung
___ none		___ hamster ___ other
___ other		___ other

S9 purchased from Sitek Research Laboratories, Rockville, Maryland
S9 mix composition: S9 fraction (10% v/v), $MgCl_2$ (10mM), Na_2HPO_4 (50mM), pH 8.0, glucose-6-phosphate (5mM), NADP (4mM), $CaCl_2$ (10mM), KCl (30mM)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

   Nonactivated conditions:
   Assay 1: 4 hour treatment (87.5, 175, 350, 700, 1,400 µg/mL), harvest at 24 hours

   Assay 2: 4 hour treatment (350, 700, 1,400 µg/mL), harvest at 24 and 48 hours

   Activated conditions:
   Assay 1: 4 hour treatment (87.5, 175, 350, 700, 1,400 µg/mL), harvest at 24 hours

   Assay 2: 4 hour treatment (175, 350, 700 µg/mL), harvest at 24 and 48 hours

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20 µg/mL).

   Properly maintained? **Yes**

   Cell line or strain periodically checked for Mycoplasma contamination? N/A

   Cell line or strain periodically checked for karyotype stability? N/A

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level for metaphase analysis was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5 mg/mL, whichever was lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected for subsequent metaphase analysis at 48-hour harvest).

2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to the test chemical or positive control for a selected time interval, then the chemical was removed and the cells were continued in culture until time of harvest

a. Cell treatment:
Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)

Assay 1: 4 hr exposure, -S9, harvest at 24 hrs
4 hr exposure, +S9, harvest at 24 hrs

Assay 2: 4 hr exposure, -S9, harvest at 24 hrs
4 hr exposure, +S9, harvest at 24 hrs
4 hr exposure, -S9, harvest at 48 hrs
4 hr exposure, +S9, harvest at 48 hrs

b. Spindle inhibition
Inhibitor used/concentration: Colcemid (0.2 μg/mL)
Administration time: 3 hours (before cell harvest)

c. Cell harvest:
Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).

d. Details of slide preparation:
Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.

e. Metaphase analysis
No. of cells examined per dose: 200
Solvent control: 200
Positive control: 100

Scored for structural: **Yes**

Scored for numerical: **No**

Coded prior to analysis: **Yes**

f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.

g. Statistical analysis: Data evaluated for statistical significance at α=0.01, using Chi-square statistics from two way contingency table.

II. REPORTED RESULTS

Analysis of dose formulations by HPLC indicated that concentrations were between 87 and 115% of target concentrations for Assay 1 and between 104 and 107% for Assay 2.

A. Preliminary cytotoxicity assay:
Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, without S9 activation, the 1,400 µg/mL concentration reduced the mitotic index to 80% of the negative control value. With S9 activation, 1,400 µg/mL reduced the mitotic index to 18% of the negative control value. The next highest dose (700 µg/mL) with S9 activation gave a relative mitotic index of 61%.

In Assay 2, without S9 activation, 1,400 µg/mL reduced the mitotic index to 31% of the negative control value 24 hours after treatment and to 52%, 48 hours after treatment. With S9 activation, the highest dose (700 µg/mL) was very toxic resulting in the presence of sparse numbers of cells. The mitotic index was reduced to 27% (based on 500 cells per replicate) of the negative control value 24 hours after treatment. High toxicity was also observed 48 hours after treatment. The next highest dose (350 µg/mL) gave a relative mitotic index of 67%, 24 hours after treatment and 64%, 48 hours after treatment.

B. Cytogenetic assay: Results are presented in Attachments 1 and 2 (study report pages 23, 24, 27-30).

In Assay 1, cultures treated with 350, 700, or 1,400 µg/mL of the test compound in the absence of S9, and 175, 350, or 700 µg/mL in the presence of S9 were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1). 2,4-D BEE caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory Assay 2, cultures treated with 350, 700, or 1,400 µg/mL of the test compound in the absence of S9, and 175 or 350 µg/mL in the presence of S9 were analyzed for chromosomal aberration frequencies at the 24-hour harvest. Cultures treated with 1,400 µg/mL of the test compound in the absence of S9 or 350 µg/mL in the presence of S9 were analyzed for chromosomal aberration frequencies at the 48-hour harvest (Attachment 2).

2,4-D BEE caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation at either harvest time. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds.

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. The reviewer agrees with the study authors that 2,4-D BEE up to 1,400 µg/mL in the absence of S9 was not clastogenic in cultured rat lymphocytes harvested 24 or 48 hours after treatment. Although cytotoxicity at 1,400 µg/mL was minimal (relative mitotic index, 80%) in Assay 1, cytotoxicity was adequate in confirmatory Assay 2 (relative mitotic index, 31%). The negative controls had comparable low frequencies of chromosome aberrations. In addition, the sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls (mitomycin C, -S9, and cyclophosphamide, +S9).

In Assay 1, in the presence of S9, as shown in Attachment 3 (study report page 22), cytotoxicity at 1,400 µg/mL was high (relative mitotic index, 18%), and consequently, this dose level was not selected for metaphase analysis. However, because the dose level selected (700 µg/mL) had marginal cytotoxicity (relative mitotic index, 61%), the observation of a low frequency of chromosomal aberrations at the cytotoxic 1,400 µg/mL dose level would have provided more convincing evidence that 2,4-D BEE is not clastogenic in this test system.

In Assay 2, in the presence of S9, as shown in Attachment 4 (study report page 26), cytotoxicity at 700 µg/mL was high resulting in too few cells for metaphase analysis. The high dose level selected for metaphase analysis (350 µg/mL) had marginal cytotoxicity (relative mitotic index, 67%). A low frequency of chromosomal aberrations in the presence of marginal cytotoxicity at 700 µg/mL (Assay 1) or 350 µg/mL (Assay 2) is not convincing evidence that 2,4-D BEE is not clastogenic in this test system. In addition, high cytotoxicity at 700 µg/mL in Assay 2 and only marginal cytotoxicity at the same dose level in Assay 1, suggests some variation in the test system or procedure between the two assays. Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of S9 activation, based on the results of this study, cannot be made.

The reviewer concludes that the results of this study do not provide sufficient evidence to consider 2,4-D BEE negative in this *in vitro* test system. It is also concluded that a new study must be conducted and the chemical be evaluated up to a reproducible cytotoxic level.

B. Study deficiencies

The following deficiencies would be expected to alter the conclusions of the study:

1. Cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis.

2. The highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity; this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay. This suggests some variation in the test system or procedure between the two assays.

Chemical analysis of the 2,4-D BEE was not included in the study report, but this deficiency would not be expected to alter the conclusions of the study.

ATTACHMENTS

TABLE 4A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D BEE Positive Control: (0.5 µg/ml MMC)

	Neg. Control[a]			350 µg/ml			700 µg/ml			1400 µg/ml			Positive control		
No. of cells scored	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 50	B 50	A+B 100
Chromatid Gaps	5	2	7	1	0	1	5	1	6	4	8	12	4	5	9
Chromosome Gaps	2	0	2	0	0	0	1	2	3	1	0	1	0	0	0
Chromatid Breaks	1	0	1	1	1	2	1	1	2	5	2	7	8	12	20
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	5	5	19	10	29
Chromosome Breaks	1	0	1	0	1	1	1	0	1	0	0	0	7	7	14
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total Aberrations	2	0	2	1	2	3	2	1	3	5	7	12	34	29	63
(excluding gaps)[b]			(1.0)			(1.5)			(1.5)			(6.0)			(63.0)
No. of cells with Aberr.	2	0	2	1	2	3	2	1	3	5	4	9	23	18	41[c]
(excluding gaps)[b]			(1.0)			(1.5)			(1.5)			(4.5)			(41.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

[a]1% DMSO

[b]Values in parentheses are percentages.

[c]Significantly (alpha<0.01) different from the negative control.

TABLE 4B

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 1

Test Chemical: 2, 4-D BEE Positive Control: (6 µg/ml CP)

	Neg. Control[a]			175 µg/ml			350 µg/ml			700 µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	1	0	1	5	0	5	2	5	7	4	1	5	1	6	7
Chromosome Gaps	0	0	0	0	0	0	3	1	4	1	0	1	2	7	9
Chromatid Breaks	2	0	2	1	0	1	3	1	4	3	0	3	10	11	21
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	18	13	31
Chromosome Breaks	0	0	0	1	0	1	0	0	0	1	0	1	9	8	17
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2
Total Aberrations	2	0	2	2	0	2	3	1	4	4	0	4	38	33	71
(excluding gaps)[b]			(1.0)			(1.0)			(2.0)			(2.0)			(71.0)
No. of cells with Aberr.	2	0	2	2	0	2	3	2	5	3	0	3	23	30	53[c]
(excluding gaps)[b]			(1.0)			(1.0)			(2.5)			(1.5)			(53.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	1	1	0	0	0	5	11	16

[a] 1% DMSO

[b] Values in parentheses are percentages.

[c] Significantly (alpha<0.01) different from the negative control.

Attachment 1 Page 2 of 2

TABLE 6A

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D BEE Positive Control: (0.5 µg/ml MMC)

	Neg. Control[a]			350 µg/ml			700 µg/ml			1400 µg/ml			Positive control		
No. of cells scored	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 100	B 100	A+B 200	A 50	B 50	A+B 100
Chromatid Gaps	0	3	3	3	2	5	1	4	5	7	7	14	3	2	5
Cromosome Gaps	0	0	0	0	5	5	2	3	5	0	0	0	1	2	3
Chromatid Breaks	0	1	1	1	1	2	1	3	4	3	1	4	13	10	23
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	0	2	2	24	16	40
Chromosome Breaks	1	1	2	0	2	2	1	0	1	0	1	1	6	2	8
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Total Aberrations	1	2	3	1	3	4	2	3	5	3	4	7	43	29	72
(excluding gaps)[b]			(1.5)			(2.0)			(2.5)			(3.5)			(72.0)
No. of cells with Aberr.	1	2	3	1	3	4	2	3	5	3	3	6	28	20	48[c]
(excluding gaps)[b]			(1.5)			(2.0)			(2.5)			(3.0)			(48.))
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	0	0	0	3	2	5

[a]1% DMSO

[b]Values in parentheses are percentages.

[c]Significantly (alpha<0.01) different from the negative control.

Attachment 2 Page 1 of 4

TABLE 6C

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 24 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D BEE Positive Control: (6 µg/ml CP)

	Neg. Control[a]			175µg/ml			350µg/ml			Positive control		
	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200	100	100	200	50	50	100
Chromatid Gaps	0	1	1	1	1	2	3	1	4	0	1	1
Chromosome Gaps	1	0	1	2	0	2	0	0	0	3	0	3
Chromatid Breaks	4	2	6	0	0	0	1	0	1	10	15	25
Chromatid Exchanges	0	0	0	0	0	0	0	0	0	23	32	55
Chromosome Breaks	1	2	3	0	0	0	2	1	3	14	4	18
Chromosome Exchanges	0	0	0	0	0	0	0	0	0	1	0	1
Total Aberrations	5	4	9	0	0	0	3	1	4	49	51	100
(excluding gaps)[b]			(4.5)			(0.0)			(2.0)			(100.0)
No. of cells with Aberr.	4	4	8	0	0	0	3	1	4	36	29	65[c]
(excluding gaps)[b]			(4.0)			(0.0)			(2.0)			(65.0)
Miscellaneous Aberr.	0	0	0	0	0	0	0	0	0	1	0	1
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0	0	0	0	9	3	12

[a]1% DMSO.
[b]Values in parentheses are percentages.
[c]Significantly (alpha<0.01) different from the negative control.

Attachment 2 Page 2 of 4

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TABLE 6B

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 48 HOURS AFTER TREATMENT IN THE ABSENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D BEE

	Neg. Control[a]			1400 µg/ml		
	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200
Chromatid Gaps	1	1	2	1	2	3
Chromosome Gaps	3	1	4	1	2	3
Chromatid Breaks	1	1	2	4	1	5
Chromatid Exchanges	0	0	0	0	0	0
Chromosome Breaks	1	0	1	0	0	0
Chromosome Exchanges	0	0	0	0	0	0
Total Aberrations	2	1	3	4	1	5
(excluding gaps)[b]			(1.5)			(2.5)
No. of cells with Aberr.	2	1	3	4	1	5
(excluding gaps)[b]			(1.5)			(2.5)
Miscellaneous Aberr.	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0

[a]1% DMSO.
[b]Values in parentheses are percentages.

Attachment 2 Page 3 of 4

TABLE 6D

RESULTS OF THE CHROMOSOMAL ABERRATION ASSAY 48 HOURS AFTER TREATMENT IN THE PRESENCE OF S-9
(The replicates are designated A & B)
ASSAY 2

Test Chemical: 2, 4-D BEE

	Neg. Control[a]			350 µg/ml		
	A	B	A+B	A	B	A+B
No. of cells scored	100	100	200	100	100	200
Chromatid Gaps	3	3	6	5	1	6
Cromosome Gaps	0	5	5	1	0	1
Chromatid Breaks	1	1	2	0	1	1
Chromatid Exchanges	0	0	0	0	0	0
Chromosome Breaks	0	0	0	0	0	0
Chromosome Exchanges	0	0	0	0	0	0
Total Aberrations	1	1	2	0	1	1
(excluding gaps)[b]			(1.0)			(0.5)
No. of cells with Aberr.	1	1	2	0	1	1
(excluding gaps)[b]			(1.0)			(0.5)
Miscellaneous Aberr.	0	0	0	0	0	0
Cells with Multiple Aberr. (5 or more aberr.)	0	0	0	0	0	0

[a]1% DMSO.
[b]Values in parentheses are percentages.

Attachment 2 Page 4 of 4

TABLE 3B

MITOTIC INDICIES (M.I.) OF RAT LYMPHOCYTE CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF THE TEST MATERIAL (2, 4-D BEE) IN THE PRESENCE OF S-9

ASSAY 1

	%M.I. Harvested 24 h after treatment		
Dose µg/ml	Replicate A	Replicate B	Average A+B
Negative control[a]	6.1	10.8	8.5
88	8.2	5.9	7.1
175	6.8	6.3	6.6
350	6.9	7.6	7.3
700	4.6	5.8	5.2
1400	2.6	0.4	1.5
Positive control[b]	3.9	4.5	4.2

relative mitotic index 61%

relative mitotic index 18%

[a]1% DMSO
[b]CP (6 µg/ml)

Attachment 3 Page 1

TABLE 5B

MITOTIC INDICIES (M.I.) OF RAT LYMPHOCYTE CULTURES TREATED WITH VARIOUS CONCENTRATIONS OF THE TEST MATERIAL (2, 4-D BEE) IN THE PRESENCE OF S-9

ASSAY 2

	%M.I. Harvested 24 h After Treatment			% M.I. Harvested 48 h After Treatment		
Dose µg/ml	Replicate A	Replicate B	Average A+B	Replicate A	Replicate B	Average A+B
Negative control[a]	5.0	5.4	5.2	5.7	3.2	4.5
175	5.0	2.4	3.7	3.7	7.3	5.5
350	2.7	4.3	3.5	2.8	3.0	2.9
700	1.6 [b]	1.2 [b]	1.4	<0.1	0.8	ND
Positive control[c]	5.5	1.3	3.4	ND	ND	ND

relative mitotic index 67%

too few cells for metaphase analysis

[a]1% DMSO
[b]Mitotic indices were based upon only 500 cells/replicate due to the presence of sparse numbers of cells.
[c]CP (6 µg/ml)
ND = Not Done

Attachment 4 Page 1 of 1

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Chemical:	**Isopropylamine 2,4-dichlorophenoxyacetat**
PC Code:	**030025**
HED File Code	**13000 Tox Reviews**
Memo Date:	**05/31/1996**
File ID:	**TX011942**
Accession Number:	**412-01-0082**

HED Records Reference Center
01/10/2001